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(54) **An Agent for the Release of Cells From Tissues or From the Surface of a Cultivation Vessel**

(57) An agent for the release of cells from tissues or from surfaces comprises 1 to 40% by weight of at least one proteolytic enzyme (such as trypsin, chymotripsin, pancreatin, pronase, elastase or colagenase) and/or at least one material forming chelates (such as bisodium salt of ethylenediaminotetraacetic acid) and 60 to 99% of auxiliary materials, including at least one material adjusting the ionic concentration to an isotonic solution (such as sodium chloride, potassium chloride or glucose).

ERRATUM

SPECIFICATION No. 2 064 543 A

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GB 2 064 543 A

SPECIFICATION

Agents for the Release of Cells from Tissues and from the Surface of Cultivation Vessels and Methods for their Manufacture

The invention relates to a method of manufacture of agents for the release of cells from tissues and from the surface of cultivation vessels, the agents being in the form of powder, granules and pressings (most frequently tablets or briquets) prepared from powdered homogenized or granulated mixtures of proteolytic enzymes, materials forming chelates and of auxiliary materials consisting mostly of inorganic salts.

For the preparation of cell suspensions from animal tissues different proteolytic enzymes are used as for instance trypsin, pancreatin, pronase, elastase, or chymotrypsin. Similarly the release of cells from the surface of cultivation vessels is accomplished by these enymes. In addition to solutions above mentioned enzymes solutions of materials forming chelates as for instance ethylenediaminetetraacetic acid, or citric acid are used. The enzymes or materials forming chelates are mostly dissolved in an isotonic solution of sodium chloride, which is suitably buffered to certain pH, at which the maximum efficiency of the enzyme prevails. The agents are manufactured for cultivation of cells and of tissues in vitro as sterile solutions or of lyophylizates for direct application (see the catalogues of Messrs Difco, USA of 1976, of Messrs Gibco, USA of 1976 and of Messrs Flow, Britain of 1977).

Trypsin and other proteolytic enzymes are not stable in aqueous solutions and are subject to proteolytic self decomposition. In the course of storage the enzymatic activity decreases and the products lose their standardized effect. When storing a 0.25 per cent solution of trypsin in a buffered physiologic solution at a pH 7.2 at —4°C, trypsin can be used at the most for 2 to 3 weeks and when storing at —20°C trypsin is stable for 6 months (see von A. Mayr, P.A. Bachmann, B. Bibrack, G. Wittmann: Virologische Arbeitsmethoden, VEB Gustav Fischer, Verlag, Jena 1974).

It is an object of this invention to eliminate this drawback by the provision of similar agents in the dry state, capable of being stored for a considerable time without substantial loss of their activity. According to this invention for the improvement of the stability of agents for the release of cells from tissues and from surfaces of cultivation vessels, enzymes, materials forming chelates and auxiliary materials consisting mostly of inorganic salts are homogenized, shaped to granules or formed on suitable press machines (at conditions mentioned later to pressings of globular, cylindrical, lenticular or other suitable shape.

The agents for the release of cells from tissues or from the surface of cultivation vessels in the form of powder, granules or pressings, particularly of tablets or briquets, contain, according to this invention, 1 to 40 per cent weight of proteolytic enzymes, particularly trypsin, chymotrypsin, pancreatin, pronase, elastase, or colagenase, or materials forming chelates, particularly bisodium salts of ethylenediaminotetraacetic acid individually or in mixtures. These agents contain furthermore 60 to 99 per cent by weight of auxiliary materials such as sodium chloride, potassium chloride, glucose, sodium dihydrogen or thiophosphate, dihydrogen potassium orthophosphate, disodium hydrogen orthophosphate, hydrogen dipotassium orthophosphate, sodium hydrogen carbonate, tris-hydroxymethylaminomethane, calcium chloride, magnesium chloride, polyethyleneglycol, polyvinylpyrolidone, methylcellulose, phenolred, N-2-hydroxyethylpiperazin-N'-2-ethansulphonic acid; N-tris hydroxymethyl-methylglycine; 2-/N-morpholine/ethane-sulphonic acid; piperazin-N,N'-bis-/2 ethane sulphonic acid; N,N'-bis-1/2-hydroxyethyl/glycine; N-tris-/hydroxy-methyl/methyl-2-aminoethane sulphonic acid; 2-bis-/hydroxyethyl/-imino-2-hydroxymethyl/-1-3-propandiol; hydroxymethyl piperazin-N'-3 propane sulphonic acid and others. The enzymes, materials forming chelates and auxiliary materials consisting predominantly of organic salts are homogenized advantageously in a fluidizing arrangement or in a planetary mixer. Into the fluid layer composed of auxiliary materials an aqueous or alcoholic solution of one or more auxiliary materials is introducedand the whole content of enzymes, of the materials forming chelates or their mixture. The mixture is dried by gas, advantageously by air at temperatures of up to 100°C, or it is first granulated and subsequently dried. According to another method the auxiliary materials are mixed in a planetary mixer and wetted by a solution of ethanol, propanol, acetone or by their mixture, in which the materials are dissolved adjusting the oncotic pressure, particularly polyethyleneglycol, polyvinylpyroline and materials indicating pH, such as phenol red. The wetted mixture is dried by gas, advantageously by air at temperatures of up to 100°C. After drying some enzyme or a material forming chelates or their mixture is added and after homogenization the granulate is pressed to pressings, advantageously to tablets or briquets at a pressure of 40 to 200 MPa. The resulting powdered homogenized or granulated material or tablets or briquets is weighed or filled in suitable bottles, sterilized by gamma radiation with a dose of 2.5 Mrad. The powdered homogenized or granulated material or tablets for the release of cells can also be sterilized after being dissolved in a given volume of redistilled water, through a Millipore filter.

An object of this invention is also the preparation of dry agents for the release of cells from tissues and from cultivation vessels in the shape of pressings (tablets, briquets or pressings of other suitable shape) or of homogenized powder and granules, which method has with respect to presently used methods of preparation

advantages in a perfect homogenization of the components, a long time stability, good solubility, in a stable standard shape and weight in case of pressings and in a simplified manipulation in preparation and transport. The obtained agents maintain all their properties for the release of cells from animal tissues or from the surfaces of cultivation vessels. An advantage of the preparation of products is their standardization in application due to the increased stability of dry enzymatic agents in the form of homogenized powder, granules or pressings (tablets, briquets). The tablets can be adjusted as to their weight so that they can be dissolved in a determined volume of water. An economical advantage of agents for the release of cells from tissues or from cultivation vessels in the shape of granules or tablets is the possibility of their long storage without the need of cooling spaces, and reduced costs on transport and packing materials. The possibility of manufacture of large amounts of products prepared by the proposed method enables the standardization of enzymatic agents to be improved. The forming of dry pressing for tissue cultures can be performed on conventional eccentric, rotating or other tabletting or briquetting machines at normal atmospheric conditions at a suitable pressure, in which the dies may be provided with a special coating (Teflon, silicon or other suitable material) after adjustment of the humidity of the starting raw material and the relative humidity of the surrounding air of 10 to 50 per cent, according to the kind of the pressed mixture of the agents for the release of cells from tissues or from cultivation vessels.

Example 1

In a fluid arrangement 400g sodiumchloride p.a., 10 g of potassium chloride p.a., 144.5g of sodium hydrogen orthophosphate p.a. and 5g of magnesium chloride with 6 H_2O p.a. are mixed. Into the fluid layer a solution of the following composition is gradually introduced: 12.5g of trypsin, 12.5g of chymotrypsin, 10g dihydrogen potassium orthophosphate p.a., 8g polyethyleneglycol with a spec. weight 6000 dissolved in 50ml of redistilled water. The obtained granulate is dried at a temperature of up to 100°C. The dried granulate can be filled at 0.6g to bottles and exposed to gamma radiation with a dose of 2.5 Mrad. After irradiation the sterile granulate is dissolved in 50ml sterile redistilled water and used for the release of cells from cultivation vessels or it is possible to press from the dried granulate tablets of the weight of 0.6g at a pressure of 98 MPa. The tablets can be sterilized by gamma radiation with an overall dose of 2.5 Mrad. One sterile tablet of a weight of 0.6g is dissolved prior to application in 50ml of sterile redistilled water.

Example 2

In a fluid arrangement 340g of sodium chloride p.a., 20g of potassium chloride p.a., 54.75g of glucose, 110g of sodium hydrogen carbonate p.a. are mixed and wetted by a solution of 40g 96% ethanol, 0.25g phenol red and 10g polyethyleneglycol with a spec. weight 6000. The obtained granulate is dried at a temperature of up to 150°C. The dry granulate is mixed with 12.5g trypsin and 12.5g chymotrypsin with a grain size of 0.125mm. From the dried mixture tablets of a weight of 0.56g are pressed at a pressure of 40 MPa. The tablets are sterilized by gamma radiation with a dose of 2.5 Mrad. One sterile tablet is prior to application dissolved in 50ml of sterile redistilled water.

Example 3

In a planetary mixer 125g of tris-hydromethylaminomethane. HCl are mixed with 25g of tris-hydromethylaminomethane and 377.5 of sodium chloride p.a. and is wetted by a mixture composed of 50g of 96 per cent ethanol, 0.25g of phenol red and 7.25g of polyvinyl pyrolidone. The obtained granulate is dried at temperatures of up to 130°C. After drying 12.5g of trypsin of a grain size 0.100mm is added to the granulate. After homogenization tablets of a weight of 0.55g and of a diameter 12mm are pressed at a pressure of 60 MPa. One tablet is prior to application dissolved in 50ml of redistilled water and the solution is prior to application sterilized by ultrafiltration through a Millipore filter 0.22μ. A sterilization by gamma radiation, as in the preceding case may also be used.

Example 4

In a fluid arrangement 400g of sodium chloride p.a., 10g of potassium chloride p.a., 144.5 of disodium hydrogen orthophosphate with 12 H_2O p.a. and 5g of magnesium chloride with 6 H_2O are mixed. In 50 ml redistilled water 12.5g of trypsin for tissue cultures, 10g of dihydrogen potassium orthophosphate p.a. and 8g polyethyleneglycol of a spec.w. 6000 are dissolved. The solution is filtered through a sintered glass filter S3. The solution is gradually introduced into the fluid layer. The obtained granulate is dried at temperatures of up to 100°C. The dried granulate is pressed to briquets of a weight 2.4g at a pressure of 200 MPa. The tablets are irradiated by gamma radiation with an overall dose of 2.5 Mrad. One sterile tablet is prior to application dissolved in 200ml of sterile redistilled water.

Example 5

In a fluid arrangement 340g of sodium chloride p.a., 20g of potassium chloride p.a. 54.75 of glucose, 100g of sodium hydrogen carbonate p.a. and 100g of bisodium salt of ethyenediaminetetraacetic acid are mixed and wetted by a solution of 0.25g of phenol red, 10g of sodium hydrogen carbonate in 30ml of redistilled water. The obtained granulate is dried at temperatures of up to 130°C. The dried granulate is mixed with 10g of trypsin and 10g of chymotrypsin of a grain size 0.125mm. 0.56g of dry homogenized powder mixture is filled in

bottles and sterilized by gamma radiation with an overall dose of 2.5 Mrad.

In the examples the abbreviation p.a. means "pro analysis, i.e. analytically pure.

Claims

1. Agents for the release of cells from tissues and from the surface of the cultivation vessels the agents being in the form of powder, granules or pressings, such as tablets or briquets, comprising 1 to 40 per cent by weight of components selected from proteolytic enzymes, such as trypsin, chymotrypsin, pancreatin, pronase, elastase, or colagenase, and from materials forming chellates, such as bisodium salt of ethylenediaminotetraacetic acid, signal and in mixtures, and further comprising 60 to 99 per cent by weight of auxiliary materials, comprising materials adjusting the ionic concentration to an isotonic solution, such as sodium chloride, potassium chloride, glucose at 0.5 to 99 per cent by weight and of buffering materials, such as sodium dihydrogen orthophospate, disodium hydrogen orthophosphate, dihydrogen potassium orthophosphate, hydrogen dipotassium orthophosphate, sodium hydrogen carbonate, tris-hydroxymethyl-aminomethane. HCl; tris-hydroxymethylaminomethane; N-2-hydroxyethylpiperazin-N'-2-ethane sulphonic acid; N-tris-hydroxymethylglycine; 2-/N-morpholine/ethane sulphonic acid; piperazin-N-N'-bis/2 ethane sulphonic acid; N,N'-bis-1/2-hydroxymethyl/glycine; N-tris-/hydroxy-methyl/methyl-2-aminomethane sulphonic acid; 2-bis-/hydroxyethyl/-imino-2 hydroxymethyl/1—3 propandiol; hydroxyethyl piperazin-N'-3 propane sulphonic acid in amounts of 1 to 99 per cent by weight.

2. Agents as claimed in claim 1, comprising in addition as auxiliary materials materials adjusting the oncotic pressure such as polyethyleneglycol, polyvinylpyrolidone, methylcellulose, hydroxyethylcellulose and other in water soluble cellulose derivatives in an amount of 0.01 to 2 per cent by weight.;

3. Agents as claimed in claim 1, comprising in addition as auxiliary materials materials indicating pH, advantageously phenol red in an amount 0.02 to 0.1 per cent by weight.

4. Agents as claimed in claim 1, comprising in addition as auxiliary materials materials increasing the enzymatic activity of proteolytic enzymes, particularly calcium chloride and magnesium chloride in an amount of 1 to 5 per cent by weight.

5. Method of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels where 60 to 90 per cent by weight of auxiliary materials (related to the weight of the resulting product) are homogenized, advantageously in a fluid arrangement to a homogeneous mixture, whereafter advantageously into the fluid layer an aqueous or alcoholic solution containing at least one auxiliary material and also a material indicating pH is added to 1 to 40 per cent by weight of materials selected from proteolytic enzymes and materials forming chelates (the percentage being related to the weight of the final product) at a pH 3 to 6, whereby the obtained mixture is dried by a gas, advantageously air at temperatures of up to 100°C.

6. Method of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels, where 60 to 90 per cent by weight of auxiliary materials is homogenized, granulated and dried, whereafter to the dried granules 1 to 40 per cent by weight (related to the weight of the final product) of materials selected from proteolytic enzymes, materials forming chelates and their mixture are added.

7. Method of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels, where 1 to 40 per cent of materials selected from proteolytic enzymes and from materials forming chelates and 60 to 90 per cent by weight of auxiliary materials are homogenized, the resulting mixture is wetted by an agent selected from acetone, ethanol isopropanol, water and their mixture, whereafter it is grnaulated and dried by a gas, advantageously by air, at temperatures of up to 100°C.

8. Method of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels, where the obtained powder product or granules are pressed to pressings such as tablets, or briquets, at pressures from 40 to 200 MPa.

9. Methods of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels, where the dry product is sterilized by gamma radiation with an overall dose of 2.5 Mrad.

10. Agents for the release of cells from tissues and from the surface of cultivation vessels, substantially as described.

11. Method of preparation of agents for the release of cells from tissues and from the surface of cultivation vessels, substantially as described.

12. An agent for the release of cells from tissues or from the surface of a cultivation vessel, the agent comprising 1 to 40 per cent by weight of at least one proteolytic enzyme and/or at least one material forming chelates, and 60 to 99 per cent by weight of auxiliary materials, including at least one material adjusting the ionic concentration to an isotonic solution.

13. An agent according to Claim 12 wherein the proteolytic enzyme is trypsin, chymotrypsin, pancreatin, pronase, elastase or colagenase.

14. An agent according to Claim 1 or 2 wherein the material forming chelates is bisodium salt of ethylenediaminotetraacetic acid.

15. An agent according to any one of the preceding claims wherein the material adjusting the ionic concentration is sodium chloride, potassium chloride or glucose.

16. An agent according to any one of the preceding claims wherein the auxiliary materials include buffering material which comprises at

least one of the following: sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, dihydrogen potassium orthophosphate, disodium hydrogen orthophosphate, hydrogen dipotassium orthophosphate, sodium hydrogen carbonate, tris-hydroxymethylaminomethane, tris-hydroxymethylaminomethane.HCl, N-2-hydroxyethylpiperazin-N'-2-ethanesulphonic acid, N-tris-hydroxymethylglycine, 2-/N-morpholine/ethane-sulphonic acid, piperazin-N, N'-bis-/2 ethane sulphonic acid, N-N'-bis-1/2-hydroxy-methyl/glycine, N-N'-bis-1/2-hydroxyethyl/glycine, N-tris-/hydroxy-methyl/methyl-2-aminomethane sulphonic acid, N-tris/hydroxymethyl/methyl-2-aminomethane sulphonic acid, 2-bis-/hydroxy-methyl/-imino-2 hydroxymethyl/1-3 propandiol, hydroxymethyl piperazin-N'-3 propane sulphonic acid or hydroxyethyl piperazin-N'-3 propane sulphonic acid.

17. An agent according to any one of the preceding claims wherein the auxiliary materials include a material adjusting the oncotic pressure.

18. An agent according to Claim 17 wherein the material adjusting the oncotic pressure includes at least one of the following: polyethyleneglycol, polyvinylpyrolidone, methylcellulose or hydroxyethylcellulose in water soluble cellulose derivatives in an amount of 0.01 to 2 per cent by weight.

19. An agent according to any one of the preceding claims wherein the auxiliary materials include a material indicating pH.

20. An agent according to Claim 19 wherein the material indicating pH is phenol red in an amount of 0.02 to 0.1 per cent by weight.

21. An agent according to any one of the preceding claims wherein the auxiliary materials include a material increasing the enzymatic activity of proteolytic enymes.

22. An agent according to Claim 21 wherein the materials increasing the enzymatic activity of proteolytic enzymes is calcium chloride and/or magnesium chloride in an amount of 1 to 5 per cent by weight.

23. A method of preparation of an agent for the release of cells from tissues or from the surface of a cultivation vessel, in which 60 to 99 per cent of auxiliary materials are homogenized to a homogeneous mixture, whereafter an aqueous or alcoholic solution containing at least one auxiliary material and also a material indicating pH is added, and 1 to 40 per cent of proteolytic enzymes and/or materials forming chelates at a pH of 3 to 6, and the obtained mixture is dried by a gas at temperatures of up to 100°C, all the percentages being by weight and related to the weight of the resulting product.

24. A method of preparation of an agent for the release of cells from tissues or from the surface of a cultivation vessel in which 60 to 99 per cent by weight of the auxilary materials are homogenized, granulated and dried, whereafter to the dried granules 1 to 40 per cent by weight (related to the weight of the final product) of at least one proteolytic enzyme and/or at least one material forming chelates are added.

25. A method of preparation of an agent for the release of cells from tissues or from the surface of a cultivation vessel in which 1 to 40 per cent of materials including at least one proteolytic enzyme and/or at least one material forming chelates, and 60 to 99 per cent by weight of auxiliary materials are homogenized, the resulting mixture is wetted by acetone, ethanol, isopropanol or water, or a mixture thereof, whereafter it is granulated and dried by a gas at a temperature of up to 100°C.

26. A method according to Claim 23 or 25 wherein the gas is air.

27. A method according to any one of Claims 23 to 26 wherein the obtained product is pressed at pressures from 40 to 200 MPa to form pressings.

28. A method according to any one of Claims 23 to 27 wherein the dry product is sterilized by gamma radiation with an overall dose of 2.5 Mrad.

29. An agent for the release of cells from tissues or from the surface of a cultivation vessel substantially as herein described with reference to Examples 1 to 5.

30. A method of preparation of an agent for the release of cells from tissues or from the surface of a cultivation vessel substantially as herein described with reference to Examples 1 to 5.

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